Reference: *Biol. Bull.*, **147**: 311–320. (October, 1974)

IDENTIFICATION AND CHARACTERIZATION OF LYSOZYME FROM THE HEMOLYMPH OF THE SOFT-SHELLED CLAM, *MYA ARENARIA* [1]

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Reactions in pelecypod molluscs to experimentally or naturally introduced non-self materials, biotic or abiotic, is primarily cellular, *i.e.*, if the foreign material is too large to be phagocytosed, it is encapsulated (see Feng, 1967; Cheng, 1967; and Cheng and Rifkin, 1970 for reviews). In the case of materials small enough to be phagocytosed, those which are digestible usually are degraded intracellularly (Tripp, 1958a, 1958b, 1960; Feng, 1959, 1965). That the vegetative cells of certain bacteria are reacted against in this manner has been documented by electron microscopy (Cheng, Cali and Foley, 1974; Cheng and Cali, 1974). Consequently, it appeared to be of interest to determine whether lysozyme and other enzymes occur in the hemolymph of several species of pelecypods and if so, to ascertain their kinetic properties. Such studies directed at the identification and characterization of lysozyme have been carried out on the serum and leucocytes of the American oyster, *Crassostrea virginica*, by Rodrick and Cheng (1974) and it has been shown that this bacteriolytic enzyme does indeed occur in both the serum and leucocytes of that mollusc. Thus, the earlier reports by McDade and Tripp (1967a, 1967b) have been confirmed and in addition, we have characterized the oyster lysozyme relative to its stability to heat, sensitivity to changes in ionic concentration, salt dependency, optimal pH range, and activity on selected species of bacteria.

The second species of pelecypod that has been studied is the soft-shelled clam, *Mya arenaria*, and in this paper is reported our studies on the lysozyme of this mollusc.

Materials and Methods

Collection of whole hemolymph

The soft-shelled clams, *M. arenaria*, used in this study were collected from the vicinity of Sandy Hook, New Jersey, and were maintained for up to 40 days in recirculating seawater tanks with a salinity of 25‰. During this period, hemolymph samples were taken from the mantle cavity, heart, and pericardial sac by use of sterile Pasteur pipettes. Because all pelecypods possess an open circulatory system, all hemolymph samples taken from these sites are assumed to be identical relative to qualitative composition.

[1] This research was supported by a grant (FD-00416–03) from the U. S. Public Health Service.

Measurement of lysozyme activity

Lysozyme (EC 3·2·1·17, N-acetylmuramide glycanohydrolase) activity in the hemolymph of *M. arenaria* was determined spectrophotometrically by a modification of the method of Shugar (1952). The modification involved using 0.2 mg/ml of dried *Micrococcus lysodeikticus* cells (Sigma, St. Louis, Missouri) and 0.1 M glycine buffer at pH 5.5. All reactions were initiated by the addition of hemolymph, *i.e.*, enzyme, and measured by use of a Gilford 240 spectrophotometer equipped with a Model 6040-A heat writing recorder. Only the initial velocities were measured and in all cases the initial velocities doubled when the amount of enzyme (hemolymph) was doubled.

The protein concentration of whole hemolymph was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) and crystalline bovine serum albumin (Sigma) was used as the standard.

Determination of pH optima

In order to determine the pH optima in the presence of several buffers, assays for lysozyme activity were carried out at various pH's in the presence of saturating substrate levels of 0.2 mg ml using the following four buffers: (1) 0.1 M glycylglycine at pH 4.0 to 9.0, (2) 0.1 M imidazole at pH 4.0 to 9.0, (3) 0.1 M Tris-HCl at pH 4.0 to 9.0, and (4) 0.1 M phosphate at pH 4.0 to 9.0. The pH of the lysozyme reaction mixture was determined before, during, and after each enzyme reaction.

Determination of end products

The lysozyme reaction mixtures were analyzed qualitatively for the presence of amino sugars (Rondle and Morgan, 1955) and reducing sugars by use of Benedict's solution (McDade and Tripp, 1967b) before, during, and after enzyme reactions.

Thermal stability of lysozyme

To determine the effect of temperature on the enzyme, samples of fresh, whole hemolymph were incubated at temperatures from 10° C to 100° C at 10° intervals for 30 minutes before being assayed for lysozyme activity.

Distribution of lysozyme

Fresh, whole hemolymph was centrifuged at 4000 × *g* for 15 minutes and the resulting pellet was homogenized in a minimal amount of 0.25 M, 0.5 M, or 0.9 M sucrose, whole hemolymph, or sea water that had been passed through a 0.22 μm millipore filter. The crude homogenate was subsequently recentrifuged for 10 minutes at 4000 × *g*. The supernatant was decanted off and recentrifuged at 10,000 × *g* for 30 minutes. This was done to pellet any organelles that had been released during homogenization. All of the supernatants and pellets were tested for lysozyme activity and the protein content of each constituent was determined.

Effect of lysozyme on bacteria

To test the specificity of the lysozyme in *M. arenaria* hemolymph, seven species of bacteria in addition to *Micrococcus lysodeitikus* were tested against this enzyme. These bacteria were *Bacillus megaterium, B. subtilis, Proteus vulgaris, Salmonella pullorum, Shigella sonnei, Escherichia coli,* and *Staphylococcus aureus.* The procedure followed is identical to that described for *M. lysodeitikus,* which involved determining alterations in optical density of the enzyme-bacteria mixtures.

Reactivation of lysozyme by salts

Freshly collected hemolymph from *M. arenaria* was dialyzed in 0.1 M glycylglycine buffer at pH 5.5 for 210 minutes after which the lysozyme activity was determined in the absence and presence of various concentrations of NaCl, KCl, and $MgCl_2$.

The sigmoid portions (first halves) of the salt reactivation curves were analyzed by use of a Hill plot to determine the minimal number of interacting binding sites for the salts during reactivation of the lysozyme.

Using the Michaelis-Menten assumption of equilibrium kinetics, *i.e.*,

$$v_0 = \frac{V_m(s)^n}{K_m + (s)^n}$$

where v_0 is the initial velocity, s is the salt concentration, and n is the number of interacting binding sites, and by taking the logarithmic form of the equation given above and arranging the logarithmic expression for the equation to fit a straight line ($y = mx + b$), the following equation is obtained:

$$\log\ (v_0/V_m - v_0) = n\ \log\ (s) - \log\ K_m$$

When the data are plotted using the coordinates log $(v_0/V_m - v_0)$ *vs.* log (s), the resulting Hill plot provides a n (= slope) equal to the minimum number of interacting binding sites (Atkinson, Hathaway and Smith, 1965).

Effect of heavy metals on lysozyme

In order to determine whether heavy metals inhibit the lysozyme from *M. arenaria* hemolymph, enzyme assays were carried out as described above but in the presence of 5 μM of zinc acetate and 0.6 μM *of* lead nitrate. In addition, since Smith and Stocker (1949) have reported that egg-white lysozyme is inhibited by sodium tartrate, we have tested the effect of 0.1 mM of sodium tartrate on the enzyme from the clam.

Results

Our results indicate that the hemolymph of *Mya arenaria* includes lysozyme activity. This conclusion is based on the finding of (1) a reduction of turbidity measured at 540 mμ when intact cell walls of several species of bacteria are placed in the hemolymph, and (2) the liberation of reducing and amine sugars by intact bacterial cell walls when placed in the hemolymph.

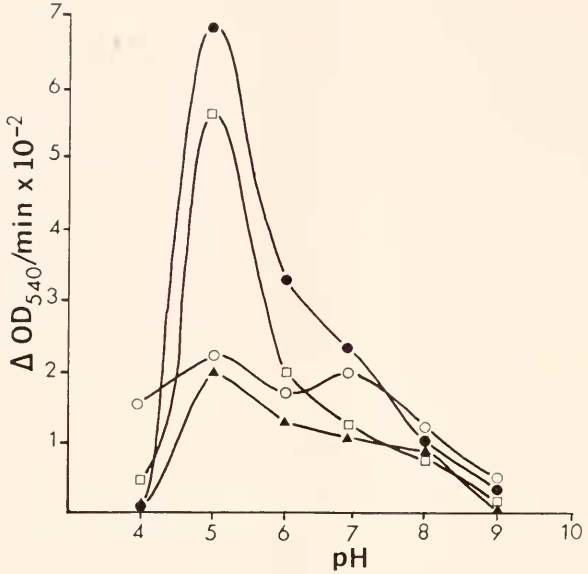

FIGURE 1. Graph showing effect of pH on the activity of the lysozyme in whole hemolymph of *Mya arenaria* using 0.1 M glycylglycine (black dots), 0.1 M Tris-HCl (circles), 0.1 M imidazole (squares), and 0.1 M phosphate (triangles) as buffers. The lysozyme activity is expressed as $\Delta OD_{540}/min \times 10^{-2}$ at 25° C.

The effect of pH on the lytic activity of whole hemolymph is shown in Figure 1. When 0.1 M glycylglycine, 0.1 M imidazole, or 0.1 M phosphate buffers are used, the optimal pH of the lysozyme has been determined to be 5.0. On the other hand, the optimal pH is 4.5 when a 0.1 M Tris-HCl buffer is employed. It is noted that a sharp peak is obtained at pH 5.0 and relatively high lysozyme activities have beeen recorded between pH 4.5 and 6.0 when 0.1 M glycylglycine and 0.1 M imidazole are used. However, low activities and a relatively broad and nonspecific pH effect is observed when 0.1 M Tris-HCl and 0.1 M phosphate buffers are employed.

As indicated in Table I, the major portion of the lysozyme activity is associated with the $4000 \times g$ supernatant while only a small amount of activity is associated with the $4000 \times g$ pellet. Similarly, when the $4000 \times g$ supernatant is recentrifuged at $10{,}000 \times g$, most, if not all, of the lysozyme activity is associated with the supernatant.

TABLE I

Distribution of lysozyme activity in the hemolymph of Mya arenaria

Sample	ΔOD_{540}/min	Specific activity
Whole hemolymph	0.012	0.009
4,000 × *g* supernatant	0.020	0.014
4,000 × *g* pellet	0.008	0.004
10,000 × *g* supernatant	0.021	0.015
10,000 × *g* pellet	0.001	undetectable

TABLE II

Lytic activity of lysozyme in whole hemolymph of Mya arenaria *on several species of bacteria. The specific activities are reported at* $\Delta OD_{540}/min/mg$ *of protein at 25°C and pH 5.5*

Bacteria	ΔOD_{540}/min	Specific activity
Micrococcus lysodeitikus	0.021	0.020
Bacillus megaterium	0.018	0.014
Bacillus subtilis	0.008	0.006
Proteus vulgaris	0.016	0.010
Salmonella pullorum	0.013	0.009
Shigella sonnei	0.011	0.008
Escherichia coli	0.009	0.007
Staphylococcus aureus	no activity	no activity

Eight species of bacteria were tested against the lysozyme in *M. arenaria* hemolymph. As indicated in Table II, the enzyme is most active in the degradation of *Micrococcus lysodeitikus*, *Bacillus megaterium*, and *B. subtilis*. There is a low level of activity against *Proteus vulgaris*, *Salmonella pullorum*, *Shigella sonnei*, and *Escherichia coli* and the lysozyme has no effect on *Staphylococcus aureus*.

That the hemolymph lysozyme of *M. arenaria* is very sensitive to dialysis is demonstrated by the reduction of its activity by 50% after 30 minutes of dialysis and the total inactivation of the enzyme after 210 minues of dialysis in 0.1 M glycylglycine (Fig. 2).

The addition of either NaCl or KCl to hemolymph that had been inactivated by dialysis restores the lysozyme activity (Fig. 3). However, the addition of both $MgCl_2$ and $FeCl_3$ has little effect on reactivation of the enzyme. It is noted that the highest lysozyme activity (ΔOD_{540}/min) is obtained with 100 mM of NaCl or KCl. Furthermore, the activation portion of the curve (first half) is sigmoidal while the deactivation portion (second half) is not sigmoidal. By plotting the data from the sigmodial activation portion as a Hill plot (Fig. 4), a straight line with a slope (n value) of 2.3 for NaCl and 2.8 for KCl is obtained. These slopes (n values) are equal to the minimum number of interacting binding sites for NaCl and KCl.

TABLE III

The effects of sodium tartrate, zinc acetate, and lead nitrate on lysozyme activity in the hemolymph of Mya arenaria

Sample	ΔOD_{540}/min	Specific activity	% inhibition
Fresh whole hemolymph	0.025	0.020	0
Fresh whole hemolymph + 0.1 mM sodium tartrate	0.008	0.006	68.0
Fresh whole hemolymph + 5 μM zinc acetate	0.011	0.008	56.0
Fresh whole hemolymph + 0.6 μM lead nitrate	0.005	0.003	80.0

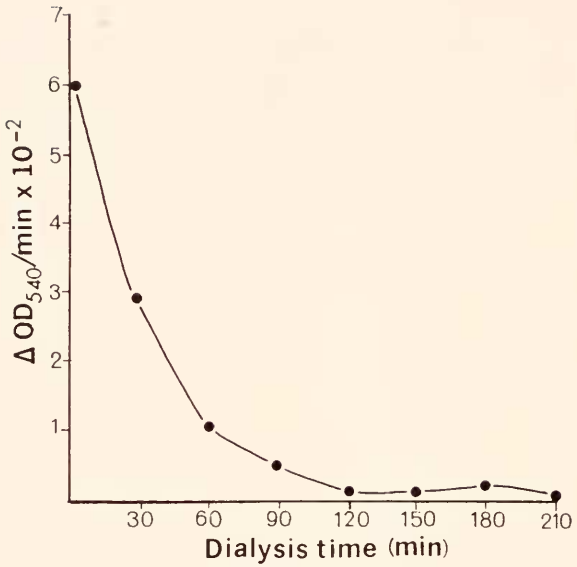

FIGURE 2. Graph showing the effect of dialysis on the lytic activity of the lysozyme in the whole hemolymph of *Mya arenaria* using 0.1 M glycylglycine at pH 5.5. The lysozyme activity is expressed as $\Delta OD_{540}/min \times 10^{-2}$ at 25° C.

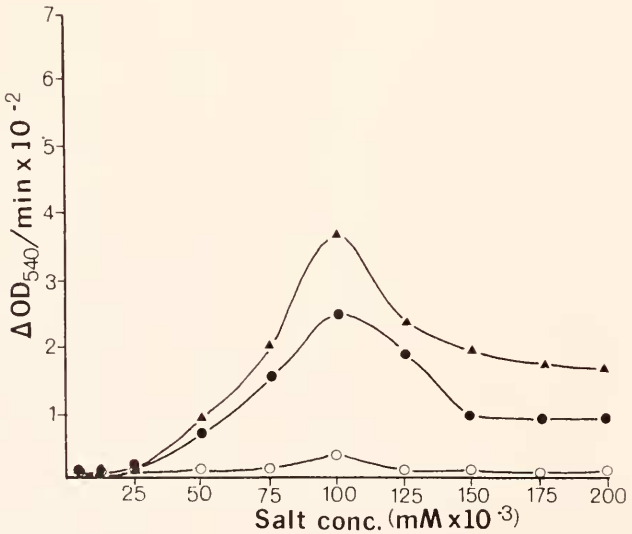

FIGURE 3. Graph showing the effect of various salt concentrations on the lytic activity of the lysozyme in the whole hemolymph of *Mya arenaria* using NaCl (triangles), KCl (black dots), and $MgCl_2$ (circles). The lysozyme activity is expressed as $\Delta OD_{540}/min \times 10^{-2}$ at 25° C and pH 5.5.

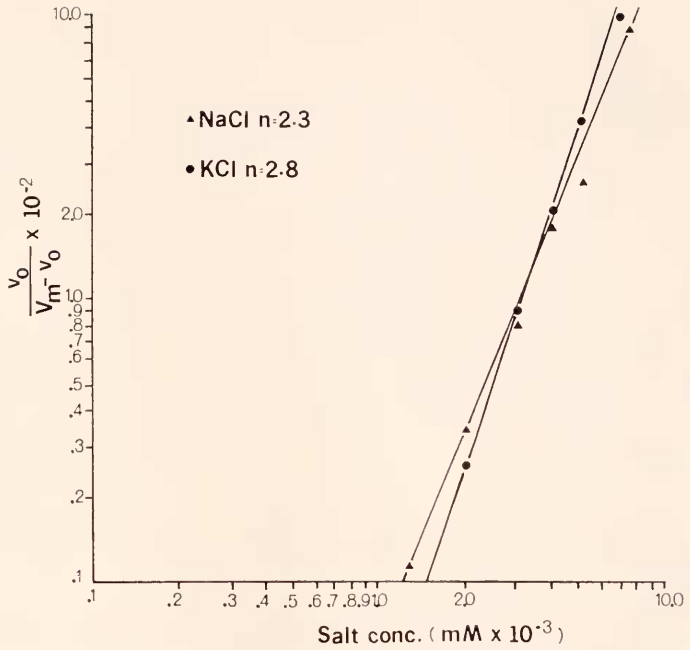

FIGURE 4. Hill plot analysis of the effect of various concentrations of NaCl (triangles) and KCl (black dots) on the lytic activity of the lysozyme in the whole hemolymph of *Mya arenaria*. The slope (n) is equal to the minimum number of interacting binding sites.

The lysozyme in the hemolymph of *M. arenaria* is extremely sensitive to small amounts of zinc acetate, lead nitrate, and sodium tartrate (Table III). Lead nitrate is approximately 10 times more effective than zinc acetate and considerably more effective than sodium tartrate in inhibiting lysozyme activity.

DISCUSSION

According to Jollès (1964), the following criteria must be met before an enzyme can be designated as lysozyme. Specifically, (1) it must catalyze the release of reducing sugars from susceptible bacteria, (2) it must cause the liberation of amine sugars and/or muramic acid from susceptible bacteria, and (3) it must cause the reduction in turbidity of intact bacterial cell walls. The enzyme that we have examined from the hemolymph of *M. arenaria* meets these criteria and therefore can be considered as lysozyme.

The lysozyme from *M. arenaria* hemolymph shares several biochemical properties with lysozymes from other sources. It is relatively heat stable, being able to withstand 75° C for 30 minutes; is salt dependent; and portrays sensitivity to alterations in salt concentration. Relative to the last characteristic, it is noted that in the absence or very low concentrations of salts, the *M. arenaria* lysozyme has essentially no effect on the cell wall of *Micrococcus lysodeikticus* as indicated by only minute amounts of reducing and amine sugars released and little, if any, decrease in turbidity of the bacterial reaction mixtures. Based on the

available data, it would appear that the salt dependency of the lysozyme is specific for monovalent cations since both KCl and NaCl are very effective in restoring enzyme activity while $MgCl_2$ is rather ineffective.

As reported, the activation of the molluscan lysozyme at low cation concentrations is sigmoidal, thus indicating the possible occurrence of preferential interacting binding sites for NaCl and KCl. A Hill plot analysis of these data has revealed that a minimum of 2.0 binding sites occur and are required for optimal activity. On the other hand, the inhibitory segments of the two salt-dependency curves (Fig. 3) are not sigmoidal and hence probably do not involve multiple reaction sites. Therefore, it may be concluded that the activation and inhibition of lysozyme activity by NaCl or KCl may represent two distinct kinetic processes. It is noted that Fitt, Dietz and Grunberg-Manago (1968), working with polynucleotide phosphorylase, have reported activation of this enzyme by low salt concentrations in a manner similar to that of the hemolymph lysozyme of *M. arenaria*. It is also noted that the hydrolysis of glycol chitin requires the presence of small amounts of salts (Rupley and Gates, 1967) and the interactions of lysozyme with chromatographic columns of neutral chitin are affected by changes in pH and ionic concentration of the medium (Davis, Heuberger and Wilson, 1969). Consequently, the overall conformation of lysozyme may require the presence of salts to affect lysis.

Phillips (1967), based on x-ray crystallography data, has shown that the lysozyme molecule includes high concentrations of salts which may suggest a role for salts in the overall confirmation of the enzyme; however, Praissman and Rupley (1968) have demonstrated that increases in ionic concentrations have only a minimal effect on the tritium-hydrogen exchange rates of lysozyme. This information could be interpreted to negate Phillips' hypothesis. Probably a more satisfactory explanation for decreased lytic activity of lysozyme in the absence of salts is that of Davis *et al.* (1969) who have postulated that the electrostatic binding between the enzyme and the bacterial cell wall occurs in either a "productive" or "unproductive" fashion similar to that of oligomers of chitin (Rupley and Gates, 1967).

It is noted that the levels of lysozyme activity are higher in both the $4000 \times g$ and $10{,}000 \times g$ supernatants than in the corresponding pellets. The latter is comprised primarily of cells and cellular constituents. Although evidence pertaining to molluscan hemolymph cells is yet unavailable, it is possible that this phenomenon reflects the release of lysozyme from cells into the serum as Wright and Malawista (1972) and Zurier, Hoffstein and Weissman (1973) have reported for mammalian leucocytes. If such is the case, then lysozyme, and possibly other lysosomal enzymes, released into the serum in molluscs may also play a role in extracellular internal defense against invading bacteria.

We wish to acknowledge Ms. Sherry Koehler for her technical assistance.

Summary

Lysozyme activity has been demonstrated in the hemolymph of the soft-shelled clam, *Mya arenaria*. When whole hemolymph is centrifuged at 4000 and 10,000 ×

g and each constituent is assayed, lysozyme activity is found to be greater in the two supernatants than in the corresponding pellets.

The lysozyme from *M. arenaria* hemolymph is salt dependent, relatively heat stabile, very sensitive to alterations in ionic concentration and the presence of heavy metals, and has an optimal pH of 5.0 when 0.1 M glycylglycine, 0.1 M imidazole, or 0.1 M phosphate buffers are employed but an optimal pH of 4.5 when 0.1 M Tris-HCl buffer is used.

A Hill plot of the data resulting from salt reactivation studies indicates that the lysozyme in *M. arenaria* hemolymph includes at least 2.0 interacting binding sites for NaCl and KCl.

When tested against a number of bacteria, the lysozyme is most active against *Micrococcus lysodeitikus* and *Bacillus megaterium*. It is less active against *Proteus vulgaris, Salmonella pullorum, Shigella sonnei, Bacillus subtilis,* and *Escherichia coli*. It is not active against *Staphyloccus aureus*.

It is suggested that the lysozyme in serum may be released from hemolymph cells.

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